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Effects of Bilirubin on Red Cell Metabolism

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Summary: Bilirubin causes certain defects in erythrocyte metabolism, mainly in glycolysis. These effects of bilirubin were studied in vitro using a relatively physiological assay medium, which consisted of heparinized whole blood. Bilirubin was added in a small quantity of NaOH solution. In the presence of bilirubin a reduced glucose consumption of red cells and an accelerated depletion in the ATP and glycerate-2,3-bisphosphate concentration was found. Bilirubin levels lower than 50 μmol/l did not influence the glucose consumption. In agreement to other investigations it was established that biblirubin causes haemolysis of red cells. ATP-deficient cells are more sensitive to this effect.

Zur Wirkung von Bilirubin auf den Erythrocytenstoffwechsel

Zusammenfassung: Bilirubin ruft bestimmte Veränderungen im Erythrocytenstoffwechsel hervor, die hauptsächlich die Glykolyse betreffen. Diese Wirkungen von Bilirubin wurden in vitro an Hand eines vergleichsweise physiologischen Versuchsansatzes untersucht, der aus heparinisiertem Vollblut bestand. In das Vollblut wurde eine kleine Menge Natronlauge mit dem darin gelösten Bilirubin einpipettiert. In Gegenwart von Bilirubin wurde eine Hemmung des Glucoseverbrauches der Erythrocyten und ein beschleunigter Abfall von ATP und Glycerat-2,3-biphosphat beobachtet. Unterhalb einer Bilirubinkonzentration von 50 μmol/l ließ sich eine Hemmung des Glucoseverbrauches nicht mehr nachweisen. In Übereinstimmung mit anderen Untersuchungen wurde festgestellt, daß Bilirubin eine Hämolyse verursachen kann. ATP-arme Erythrocyten sind gegenüber dieser Wirkung von Bilirubin besonders empfindlich.

Introduction

An elevated level of unconjugated serum bilirubin is most frequently found during the newborn period. A rapid increase of bilirubin and high values in many patients are caused by a serologic incompatibility between mother and child. If an incompatibility cannot be confirmed, transitory hyperbilirubinaemia is the most frequently assumed explanation for the elevated bilirubin concentrations.

The question of whether bilirubin itself participates in the etiology of transitory hyperbilirubinaemia is of great interest. As early as 1966 *Cheung* et al. demonstrated that bilirubin interferes with red cell metabolism (1). When red cells are incubated with bilirubin they consume less glucose and the ATP concentration drops more rapidly compared with samples incubated in the absence of bilirubin. These results were confirmed by later authors, who additionally reported an inhibition of membrane ATPase and a haemolysis of red cells induced by bilirubin (2, 3).

So far, however, published studies concerning the effects of bilirubin on red cells in vitro are difficult to compare with those reported in vivo. In all studies bilirubin was dissolved in a buffer and red cells were added. Bilirubin, however, is not soluble in aqueous solutions at a physiological pH value. The pH has to be increased to a value of about 8.0. This is accompanied by an inevitable increase in red cell glycolysis (4, 5). Furthermore the suspension of red cells in a buffer results in a reduced pentose phosphate pathway (6). These disadvantages can easily be avoided by dissolving bilirubin in NaOH and adding a small amount of this solution directly into whole blood. The necessary amount of NaOH can be limited to such a small quantity that the increase in the pH is minimal. Using this model the effects of bilirubin on red cell integrity, glucose consumption and the content of organic phosphates were examined.

Methods

Bilirubin was dissolved in 1 ml NaOH (0.1 mol/l) and 10 μl of this solution were added to 2 ml of heparinized blood from a healthy adult donor. The sample was incubated without further

additives at 37 °C in the dark. A blank sample from the same donor was prepared exactly in the same way, containing the same amount of NaOH without bilirubin. In both samples the leukocytes were removed by discarding the buffy coat layer after centrifugation. The quantity of bilirubin dissolved in the NaOH was chosen so that the final bilirubin concentrations were 70–80 µmol/l.

At intervals of 2 hours the contents of ATP (7), ADP (8), AMP (8), glycerate-2,3-bisphosphate (9), and glucose (10) were determined in both samples by standard laboratory methods.

Bilirubin-induced haemolysis was measured by determination of the free haemoglobin in the plasma. 200 µl of the supernatant were added to 5 ml of cyanhaemiglobin solution and the absorbance was read against pure cyanhaemiglobin. Bilirubin determination were performed according to the method of *Jendrassik & Gróf* (11).

To prove a significant difference between the bilirubin-containing sample and the blank sample the paired t-test was used. A significant difference was assumed in those investigations when p was found to be less than 0.05.

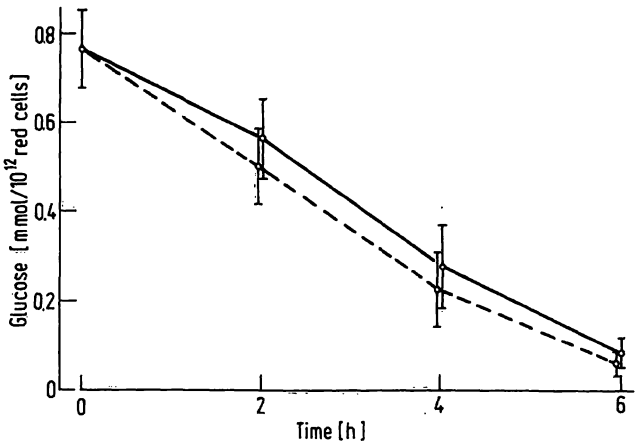

Fig. 1. Mean value and standard deviation of the glucose concentration in the bilirubin containing sample (——) and the blank sample (– – –) at different intervals. The difference is significant at 4 and 6 hours.

Results

Glucose consumption of red cells

The glucose consumption of the erythrocytes was found to be lowered in the bilirubin-containing sample, compared to the blank sample (fig. 1). The effect of bilirubin is significant after an incubation time of 4 hours ($t_{paired} = 5.8$), and 6 hours ($t_{paired} = 9.2$). The final bilirubin concentrations in these experiments ranged from 70–80 µmol/l. The increase in the pH after addition of the bilirubin-containing NaOH to the heparinized blood sample was found to be minimal. In five experiments the initial pH values ranged from 7.38 to 7.41, after addition of the NaOH from 7.43 to 7.46.

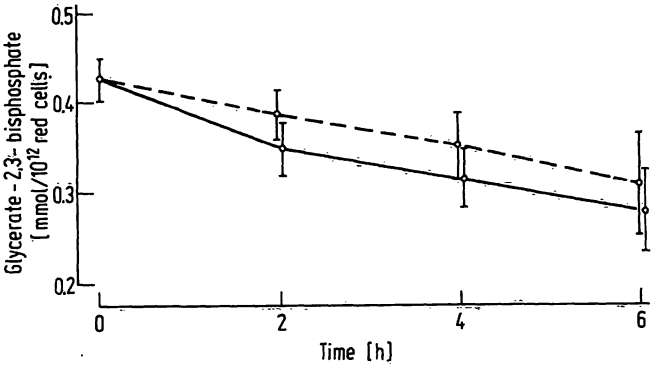

Fig. 2. Mean value and standard deviation of the glycerate-2,3-biphosphate concentration in red cells in the presence of bilirubin (——) as compared to a blank sample (– – –). In the bilirubin containing sample glycerate-2,3-bisphosphate is significantly lower at each time of investigation.

Glycerate-2,3-bisphosphate, ATP, ADP, and AMP levels

Glycerate-2,3-biphosphate as well as ATP decrease significantly and more rapidly in the presence of bilirubin (fig. 2 and 3). These results are significant at each time period of the investigation (t_{paired} for glycerate-2,3-biphosphate at 2, 4, and 6 hours = 19.9, 42.4, 6.7; t_{paired} for ATP = 9.8, 10.5 and 6.2). ADP and AMP levels, on the other hand, are not influenced by bilirubin. Table 1 gives the concentrations of these organic phosphates. No reliable differences can be recognized.

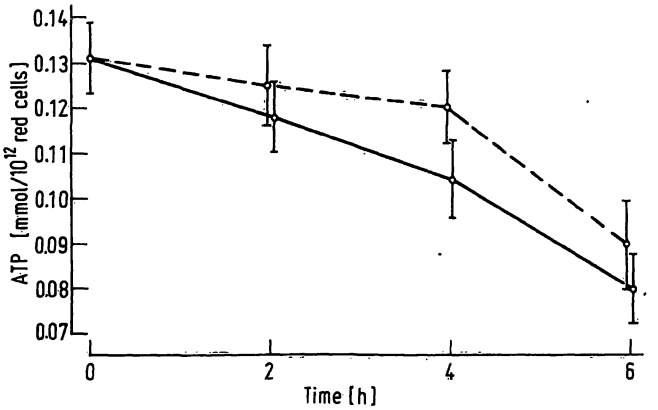

Fig. 3. Mean value and standard deviation of the ATP concentration in red cells in the presence of bilirubin (——) as compared to a blank sample (– – –). ATP levels are lower in the bilirubin-containing assay.

Bilirubin-induced haemolysis of red cells and ATP levels

Bilirubin induces haemolysis of red cells at a concentration between 70–80 µmol/l after a period of 4 hours. Haemolysis increases rapidly during further investigation (fig. 4). The blank sample shows a minimal haemolysis after an incubation time of 8 hours.

The regression line is described by the equation $y = 1.92e^{0.37x}$. The relationship of time to haemolysis is significant ($r = 0.531$, $p < 0.001$, $n = 24$). Figure 5 shows the relationship of the measured ATP concentration in the red cells versus the degree of haemolysis. The significance can also be confirmed between these two parameters ($y = 12.6e^{-0.011x}$, $r = -0.676$, $p < 0.01$, $n = 24$).

Tab. 1. ADP and AMP concentrations in whole blood containing bilirubin in a concentration of 70–80 µmol/l at different periods of incubation. The results are compared to a blank sample, which contains no bilirubin. No reliable differences between the two assays can be recognized.

	Blank sample				Bilirubin-containing sample			
Incubation time (h)	0	2	4	6	0	2	4	6
ADP (µmol/10^{12} red cells)								
$\bar{x}$	11.4	9.5	9.0	9.5	11.4	9.4	9.2	14.4
± s	1.4	3.1	4.5	6.0	1.4	2.6	2.3	8.2
AMP (µmol/10^{12} red cells)								
$\bar{x}$	4.9	4.8	4.4	5.9	4.9	4.6	5.7	6.0
± s	0.7	0.8	1.5	3.7	0.7	0.8	1.1	4.0

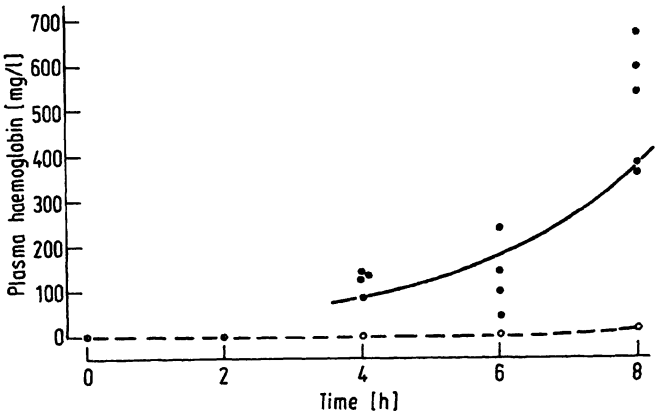

Fig. 4. Relationship of the bilirubin-induced haemolysis versus time. After an incubation period of four hours bilirubin causes haemolysis of red cells, which cannot be demonstrated in the blank sample until eight hours, and then to a far lesser extent (• = bilirubin containing sample, o = sample free of bilirubin).

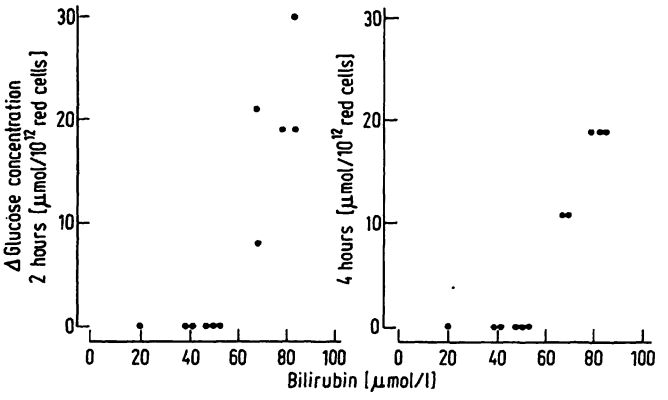

Fig. 6. Minimal effective bilirubin concentration which is able to inhibit glucose consumption of red cells. Below a bilirubin concentration of 50 µmol/l there is no difference between the test and a bilirubin free sample; bilirubin concentrations greater than 60 µmol/l result in a decreased glucose consumption. The vertical axis of the figure shows the difference in the glucose concentration between the bilirubin containing and bilirubin free sample.

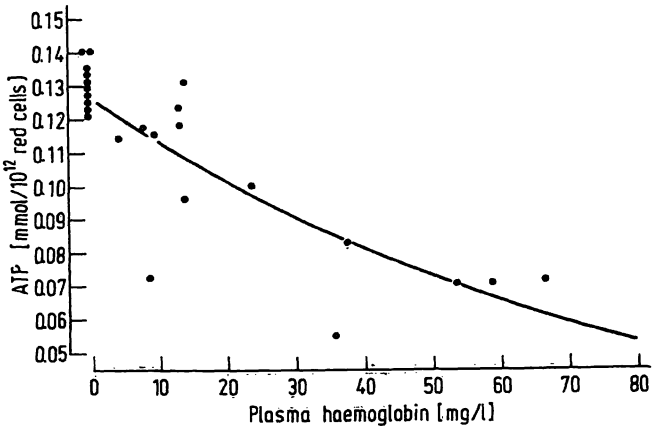

Fig. 5. Relationship of bilirubin-induced haemolysis versus ATP levels of the red cells. The relationship is significant (see text).

Minimal effective bilirubin concentration

To determine the lowest bilirubin concentration which is able to inhibit erythrocyte metabolism, the glucose consumption of red cells was measured at different bilirubin concentrations. Figure 6 shows the results of these experiments. The difference in the glucose concentration between the bilirubin-containing sample and the blank sample is registered on the vertical axis, while the bilirubin level is given on the horizontal axis. Results are given separately for an incubation time of 2 and 4 hours. Bilirubin concentrations below 50 µmol/l do not inhibit the glucose consumption. At values above 60 µmol/l it is reduced, and this can be seen from the higher glucose concentration in the bilirubin-containing blood sample. Thus a bilirubin concentration between 50 and 60 µmol/l has to be regarded as critical for erythrocyte metabolism.

Discussion

Our study confirms earlier reports (1) that bilirubin inhibits the glucose consumption of red cells and causes an ATP depletion. Additionally an accelerated decrease in the glycerate-2,3-bisphosphate concentration was observed. These effects can be demonstrated at comparatively low bilirubin concentrations and in the

presence of physiological concentrations of all plasma components. The ATP and glycerate-2,3-bisphosphate depletion can be explained by the inhibited glucose consumption, as the synthesis of both of these organic phosphates depends upon an adequate glucose supply to the red cell.

The reduced glucose consumption can be explained either by an inhibition of red cell glycolysis or by a reduced glucose uptake through the membrane. Although an inhibition of glycolysis is not excluded, the impairment of the glucose uptake appears more likely. Thus *Girotti* et al. (12) demonstrated, in the presence of bilirubin, slight modifications in the red cell membrane proteins, some of which are important for active glucose transport through the membrane (13). Inhibition of the membrane ATPase also indicates the primary action of bilirubin at the membrane.

The inhibition of glucose consumption in red cells is caused by bilirubin concentrations, which can easily be found in many newborns during the first days of life. The critical bilirubin level has been estimated to be about 60 μmol/l. This is far below the value determined by *Cheung* et al. (1). An advantage of our experiments is the presence of all normal plasma components during the incubation period. This is especially important for albumin because of its bilirubin binding properties (14). However, even at low concentrations of bilirubin a steady state exists between the binding to albumin and the binding to the red cells (15). In accordance with earlier investigations it was observed that bilirubin induces haemolysis of red cells (2, 3, 16). The degree of haemolysis depends upon the time bilirubin acts on the erythrocytes. In an earlier investigation we found a significant relationship between bilirubin-induced haemolysis and the ATP concentration within the red cells (16). This relationship could be confirmed by incubating ATP depleted red cells with inosine, thus increasing the ATP level and reducing haemolysis. Although this study was performed by suspending red cells in a buffer, our present results support this relationship.

The inhibitory effects of bilirubin on red cell glucose consumption and its haemolytic effect appear to be significant especially during the newborn period. As recent investigations have recognized haemolysis as an important factor in newborns suffering from transitory hyperbilirubinaemia (17, 18), it is speculated that similar effects of bilirubin to the described in vitro results could be of importance for the clinical course of this abnormality.

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